Interaction of the p62 subunit of dynactin with Arp1 and the cortical actin cytoskeleton

Jorge A. Garces*, Imran B. Clark†, David I. Meyer† and Richard B. Vallee*

Targeting of the minus-end directed microtubule motor cytoplasmic dynein to a wide array of intracellular substrates appears to be mediated by an accessory factor known as dynactin [1–4]. Dynactin is a multi-subunit complex that contains a short actin-related protein 1 (Arp1) filament with capZ at the barbed end and p62 at the pointed end [5]. The location of the p62 subunit and the proposed role for dynactin as a multifunctional targeting complex raise the possibility of a dual role for p62 in dynein targeting and in Arp1 pointed-end capping. In order to gain further insight into the role of p62 in dynactin function, we have cloned cDNAs that encode two full-length isoforms of the protein from rat brain. We found that p62 is homologous to the nuclear migration protein Ropy-2 from *Neurospora* [6]; both proteins contain a zinc-binding motif that resembles the LIM domain of several other cytoskeletal proteins [7]. Overexpression of p62 in cultured mammalian cells revealed colocalization with cortical actin, stress fibers, and focal adhesion sites, sites of potential interaction between microtubules and the cell cortex [8,9]. The p62 protein also colocalized with polymers of overexpressed wild-type or barbed-end-mutant Arp1, but not with a pointed-end mutant. Deletion of the LIM domain abolished targeting of p62 to focal-adhesion sites but did not interfere with binding of p62 to actin or Arp1. These data implicate p62 in Arp1 pointed-end binding and suggest additional roles in linking dynein and dynactin to the cortical cytoskeleton.

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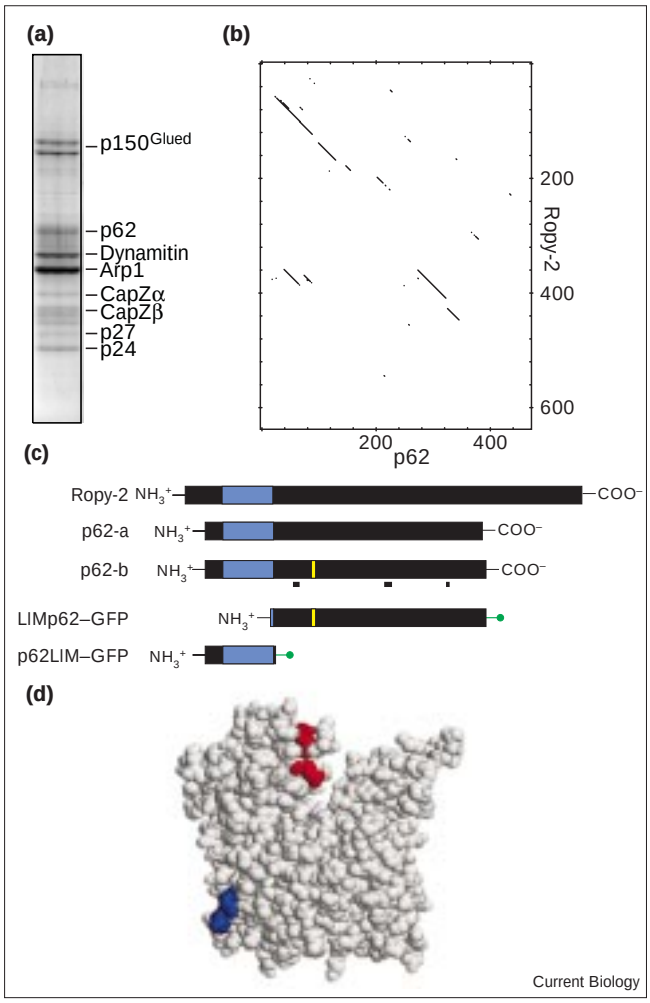
Results and discussion

Full-length rat brain cDNAs encoding p62 were cloned using amino-terminal amino acid sequence from three tryptic peptides of bovine brain p62 (LIEYYQQLAQK, IQMVAVNYIPEVR and VGIFIK in the single-letter amino acid code; Figure 1a,c). Sequence analysis revealed the existence of two isoforms of predicted size 53 and 52 kDa that differ by a seven amino acid insertion at amino acid 180. The p62 sequences are homologous throughout to the product of the *Neurospora crassa ropy-2* gene [6], a member of a class of genes involved in nuclear distribution that also includes genes encoding other dynein and dynactin components [10]. Both p62 and Ropy-2 have an amino-terminal cysteine-rich LIM domain (Figure 1b,c) that may serve as a metal-binding motif and mediate interactions with other proteins [7].

Our previous work revealed that p62 cofractionates exclusively with the 18S dynactin peak, as judged by sucrose density centrifugation [1]. In agreement with the pattern observed for other subunits of dynactin, p62 was found to localize to spindle poles and kinetochores during mitosis and to small, dispersed punctate structures during interphase (see Supplementary material). Although dynactin is found at some discrete sites within the cell, we did not observe clear localization to structures, such as the Golgi apparatus, the distribution of which is controlled by dynactin and dynein, perhaps because of the transitory nature of the interaction. For this reason, we overexpressed Myc- and green fluorescent protein (GFP)-tagged versions of p62 in COS-7 cells and examined their distribution pattern. Like endogenous p62, the overexpressed protein was observed to accumulate at spindle poles (see Supplementary material) and kinetochores (data not shown). The most striking feature of both overexpressed p62 isoforms, however, was their recruitment to actin-rich structures, including cortical actin and stress fibers (Figure 2a–e). The association of p62 with these structures was discernible in unextracted cells fixed in either methanol or paraformaldehyde, but became more clearly evident following detergent extraction. To test whether p62 and actin would interact *in vitro*, we expressed p62–Myc using a reticulocyte lysate system and assayed for cosedimentation with filamentous (F)-actin. Most of the recombinant p62–Myc pelleted in the presence of skeletal muscle F-actin (Figure 2f), in contrast to a control protein (luciferase), which remained entirely in the supernatant (data not shown).

Another notable aspect of the p62–Myc distribution was its preferential binding to stress-fiber ends (Figure 2a–c), which coincided with focal-adhesion sites as shown by codistribution with vinculin (Figure 3a–c). With decreasing p62–Myc or p62–GFP expression levels, this feature became more prominent relative to staining at stress fibers

Figure 1



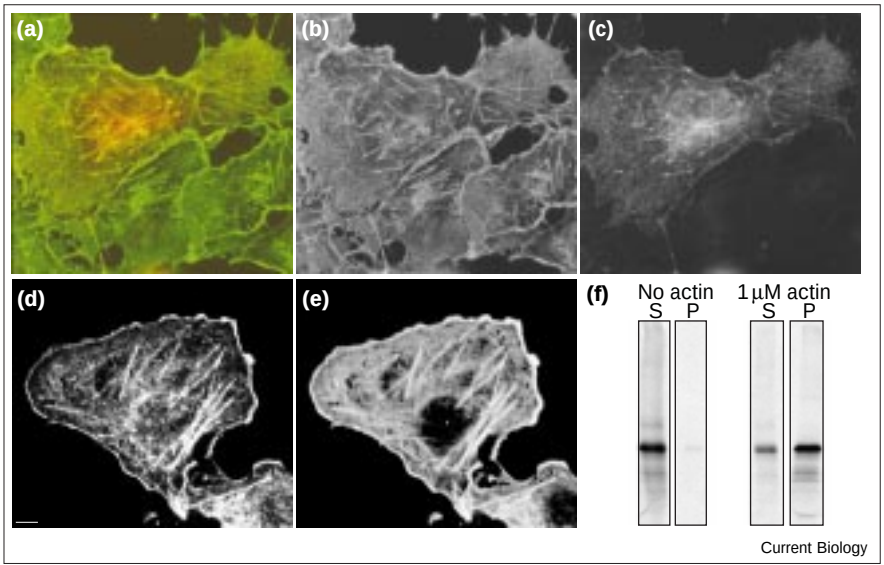
Cloning of the p62 subunit of dynactin and diagram of p62 and Arp1 expression constructs. (a) SDS–PAGE gel lane of purified dynactin. (b) A dotplot matrix analysis using the COMPARE function of the GCG software package to evaluate the similarity between p62 and Ropy-2 reveals conservation within the amino-terminal LIM domain and within the carboxy-terminal half of the two polypeptides. The window size was 30 amino acids and the stringency was a minimum of 16.5 matches per window. (c) Domains of p62 and Ropy-2 and deletion constructs. The LIM domain is in blue and the seven amino acid insertion in p62-b is in yellow. GenBank accession numbers: p62-a, AF192493; p62-b, AF192494. Bars beneath p62-b indicate the location of matching bovine peptides. (d) A RasMol-generated frontal view of β-actin, which was used to predict the approximate locations of the double missense mutations engineered into Arp1 [14]. Glu210Ala and Glu212Ala substitutions at the pointed end are in red and the Lys327Ala and Arg329Ala substitutions at the barbed end are in blue.

and cortical actin, suggesting that the interaction with focal adhesions is of higher affinity. Several LIM-domain-containing proteins have been shown to associate with the actin cytoskeleton and are thought to play a role in organization of adhesion plaques and microfilaments [7,11]. A LIM domain within paxillin has been identified as the principal focal-adhesion targeting element for the protein and its deletion resulted in the loss of focal-adhesion labeling [12]. We found that removal of the amino-terminal LIM-domain-containing portion of p62 similarly abolished focal-adhesion staining (Figure 3d–f) but, interestingly, had no effect on stress-fiber localization. The amino-terminal LIM-domain-containing fragment formed cytoplasmic aggregates when expressed on its own, suggesting improper folding (Figure 4g′).

Some of these interactions might reflect the normal association of p62 with the Arp1 filament core of the dynactin complex. To examine the molecular basis of the interaction between Arp1 and p62, we cotransfected COS-7 cells with p62 and either wild-type Arp1 or double missense mutants of Arp1 designed to interfere with interactions at the barbed-end or pointed-end surface of the molecule (Figure 1d). The wild-type and mutant forms of Arp1 formed large filamentous cables and other aggregates of less-well-defined morphology in transfected cells as reported in other studies [13,14]. Full-length p62 was found to codistribute with the cables of wild-type or barbed-end-mutant Arp1 (Figure 4a,b) but no colocalization with the pointed-end mutant of Arp1 was detected (Figure 4c). To test for proper folding of the pointed-end Arp1 mutant, we co-expressed it with the $p150^{Glued}$ subunit of dynactin. We observed a clear codistribution of the dynactin $p150^{Glued}$ subunit with barbed-end, pointed-end (Figure 5e,f), and wild-type Arp1 (data not shown). In contrast, no colocalization was observed with β-galactosidase (Figure 5d). Together, these data strongly support a specific interaction between p62 and the pointed-end surface of Arp1. Whether the interaction is direct or indirect remains to be seen. Because most of the overexpressed p62 was recruited to the Arp1 cables in the co-overexpression study, however, it seems unlikely that an endogenous intermediary factor could be responsible. In this case, p62 may represent a novel pointed-end Arp1 binding protein. We note that, as this work neared completion, p62 was reported to exist in a complex with a novel actin-related protein, Arp11, and two smaller polypeptides [15]. We suggest that p62 and Arp11 may constitute a bivalent Arp1 pointed-end binding complex, comparable to the F-actin-capping Arp2/3 complex [16].

Deletion of the LIM domain from p62 had no effect on its codistribution with Arp1 (Figure 4h). This result serves to further distinguish the binding properties of the two regions of p62 we have delineated: the carboxy-terminal region of p62 that can interact with both Arp1 and actin-containing structures, and the amino-terminal LIM-containing region responsible for focal-adhesion targeting. Presumably, the LIM domain interacts with other focal-adhesion proteins rather than with actin, but further work will be needed to explore this issue.

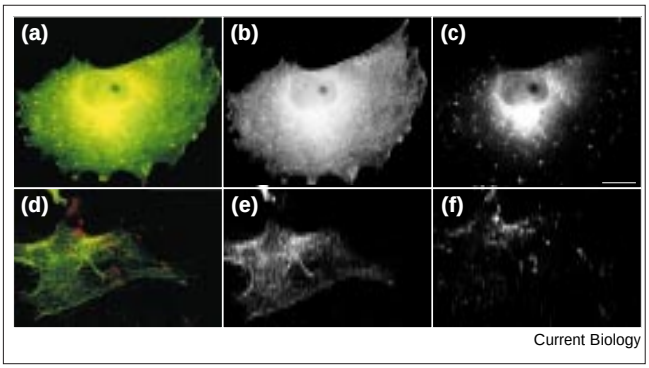
Figure 2



Behavior of p62 *in vivo* and *in vitro*. **(a–e)** COS-7 cells were transfected with p62–Myc and pre-extracted after 36 h with PNEM buffer (20 mM PIPES (pH 6.9), 150 mM NaCl, 2 mM EGTA, 5 mM $MgCl_2$) containing 0.5% Triton X-100 for 30 sec, and then (d,e) fixed in methanol at –20˚C for 8 min or (a–c) 3.2% formaldehyde for 15 min. Double labeling was performed using (c,e) an anti-actin antibody and (b,d) an anti-Myc antibody to detect p62. (a) is an overlay of (b) and (c) in which actin appears in green. Labeling of actin with rhodamine-conjugated phalloidin or expression of p62–GFP yielded identical results. The scale bars represent 5 μm. **(f)** p62–Myc was *in vitro* translated in the presence of [^{35}S]Met, incubated in the presence or absence of 1 μM skeletal muscle actin and centrifuged. Following three washes in binding buffer, the supernatant (S) and pellets (P) were analyzed by SDS–PAGE and subjected to autoradiography.

Existing evidence suggests that different dynactin subunits may have distinct targeting roles. For example, dynamitin mediates the association of the complex with the kinetochore via a direct interaction with ZW10 [1,4], whereas p150^{Glued} targets dynactin to microtubule plus ends [9]. It has also been proposed that Arp1 may mediate the binding of dynactin to the Golgi apparatus through an association with a spectrin-linked cytoskeletal network [13]. Our results suggest a potential role for p62 in targeting dynactin and dynein to focal-adhesion sites. This possibility is intriguing in view of recent evidence for crosstalk between microtubule plus ends and these cortical structures [8]. It has been shown that microtubules promote the remodeling of focal adhesions [17], but the nature of the communication between microtubule ends and focal adhesions is unclear. However, these are the precise microtubule sites where dynactin is found, as deduced from its colocalization with the microtubule end-binding protein CLIP-170 [9], which has been observed to associate only with those microtubules undergoing active elongation [18]. Whether dynactin is involved in focal-adhesion remodeling is unknown, but p62 now becomes a significant candidate for mediating such an interaction.

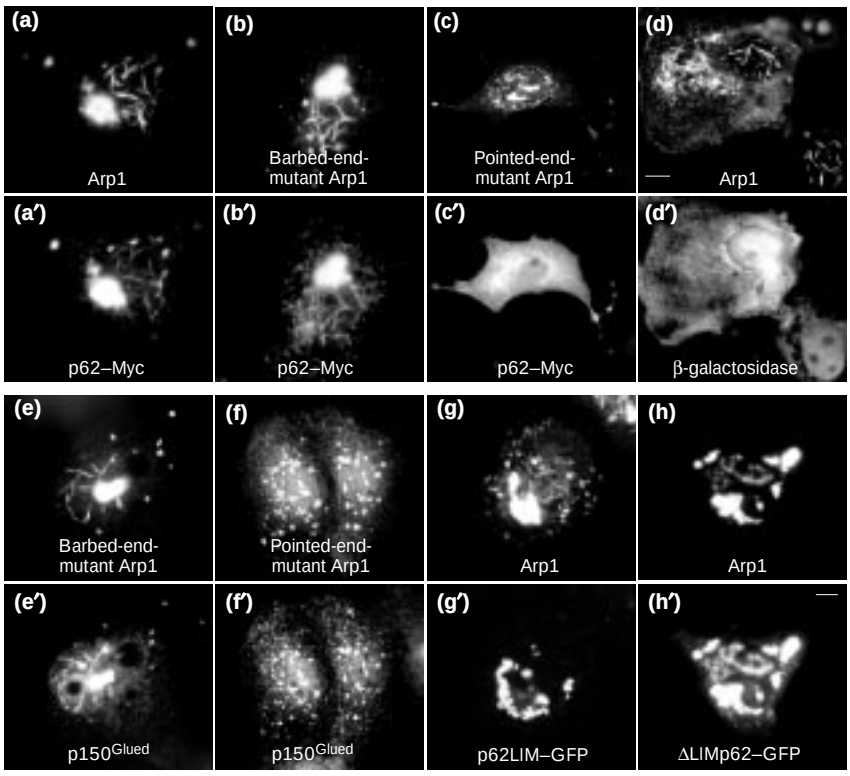
Figure 3



Targeting of p62 to focal adhesions through the amino-terminal LIM domain. **(a–c)** COS-7 cells transfected with p62–GFP were simultaneously fixed and extracted in PBS containing 3.2% formaldehyde and 0.5% Triton X-100 for 15 min and labeled with anti-vinculin antibody (a) Overlay of (b) p62–GFP fluorescence and (c) anti-vinculin staining (Texas Red). **(d–f)** Cells expressing ΔLIMp62–GFP were pre-extracted in PNEM containing 0.5% Triton X-100 for 30 sec and then fixed in 3.2% formaldehyde for 15 min. (d) Overlay of (e) ΔLIMp62–GFP fluorescence and (f) anti-vinculin staining. The p62–Myc constructs were observed to localize in a similar manner. The scale bars represent 10 μm.

The physiological significance of the association of p62 with stress fibers and cortical actin is less certain. As noted above, this behavior is mediated by the Arp1-binding portion of p62 and could reflect redistribution of excess p62 to lower-affinity sites within the cell. It is also possible, however, that it represents another aspect of microtubule–cortical interactions. Dynactin and cytoplasmic dynein have been found at the cell cortex of dividing MDCK cells [19], as well as in *Caenorhabditis elegans* embryos [20], and could be involved in producing tension at the plus ends of mitotic spindle microtubules. Interestingly, a change in the intensity of actin staining at hyphal apices and subapical actin plaques was reported in the *ropy-2* mutants of *Neurospora* [6], suggesting an involvement of the protein in the organization of these structures. The molecular basis for the interaction between dynein, dynactin, and the cell cortex is unresolved, but p62 could conceivably represent an intermediary in the interaction between microtubules and the cortical-actin cytoskeleton.

Figure 4



Co-expression of p62 with mutant and wild-type Arp1 implicates p62 in pointed-end Arp1 binding. COS-7 cells were cotransfected with full-length p62–Myc (shown here) or p62–GFP (data not shown) or β-galactosidase or p62LIM–GFP or ΔLIMp62–GFP along with either wild-type or mutant Arp1. Cells were fixed with 3.2% formaldehyde in PNEM buffer followed by incubation in 0.5% Triton X-100 for 2 min. (a,d,g,h) Wild-type Arp1, (b,e) barbed-end-mutant Arp1 and (c,f) pointed-end-mutant Arp1 were all detected using the A27 antibody [14] followed by Texas-Red-conjugated secondary antibody. Distribution of overexpressed (a′–c′) p62–Myc, (d′) β-galactosidase, and (e′,f′) the p150^{Glued} subunit of dynactin were observed by indirect immunofluorescence using monoclonal antibodies to the Myc epitope, β-galactosidase or p150^{Glued} followed by goat anti-mouse Immunoglobulin G conjugate. (g′) p62LIM–GFP and (h′) ΔLIMp62–GFP fluorescence. The scale bars represent 10 μm.

Supplementary material

Supplementary material including an alignment of p62 variants with Ropy-2 is available at http://current-biology.com/supmat/supmatin.htm.

References

1. Echeverri CJ, Paschal BM, Vaughan KT, Vallee RB: Molecular characterization of the 50-kD subunit of dynactin reveals function for the complex in chromosome alignment and spindle organization during mitosis. *J Cell Biol* 1996, 132:617-633.
2. Burkhardt JK, Echeverri CJ, Nilsson T, Vallee RB: Overexpression of the dynamitin (p50) subunit of the dynactin complex disrupts dynein-dependent maintenance of membrane organelle distribution. *J Cell Biol* 1997, 139:469-484.
3. Vallee RB, Sheetz MP: Targeting of motor proteins. *Science* 1996, 271:1539-1544.
4. Starr DA, Williams BC, Hays TS, Goldberg ML: ZW10 helps recruit dynactin and dynein to the kinetochore. *J Cell Biol* 1998, 142:763-774.
5. Schafer DA, Gill SR, Cooper JA, Heuser JE, Schroer TA: Ultrastructural analysis of the dynactin complex: an actin-related protein is a component of a filament that resembles F-actin. *J Cell Biol* 1994, 126:403-412.
6. Vierula PJ, Mais JM: A gene required for nuclear migration in ***Neurospora crassa*** codes for a protein with cysteine-rich, LIM/RING-like domains. *Mol Microbiol* 1997, 24:331-340.
7. Dawid IB, Breen JJ, Toyama R: LIM domains: multiple roles as adapters and functional modifiers in protein interactions. *Trends Genet* 1998, 14:156-162.
8. Kaverina I, Rottner K, Small JV: Targeting, capture, and stabilization of microtubules at early focal adhesions. *J Cell Biol* 1998, 142:181-190.
9. Vaughan KT, Tynan SH, Faulkner NE, Echeverri CJ, Vallee RB: Colocalization of cytoplasmic dynein with dynactin and CLIP-170 at microtubule distal ends. *J Cell Sci* 1999, 112:1437-1447.
10. Plamann M, Minke PF, Tinsley JH, Bruno KS: Cytoplasmic dynein and actin-related protein Arp1 are required for normal nuclear distribution in filamentous fungi. *J Cell Biol* 1994, 127:139-149.
11. Schmeichel KL, Beckerle MC: Molecular dissection of a LIM domain. *Mol Biol Cell* 1997, 8:219-30.
12. Brown MC, Perrotta JA, Turner CE: Identification of LIM3 as the principal determinant of paxillin focal adhesion localization and characterization of a novel motif on paxillin directing vinculin and focal adhesion kinase binding. *J Cell Biol* 1996, 135:1109-1123.
13. Holleran EA, Tokito MK, Karki S, Holzbaur EL: Centractin (ARP1) associates with spectrin revealing a potential mechanism to link dynactin to intracellular organelles. *J Cell Biol* 1996, 135:1815-1829.
14. Clark IB, Meyer DI: Overexpression of normal and mutant Arp1(alpha) (centractin) differentially affects microtubule organization during mitosis and interphase. *J Cell Sci* 1999, 112:3507-3518.
15. Eckley DM, Gill SR, Melkonian KA, Bingham JB, Goodson HV, Heuser JE, *et al.*: Analysis of dynactin subcomplexes reveals a novel actin-related protein associated with the Arp1 minifilament pointed end. *J Cell Biol* 1999, 147:307-320.
16. Mullins RD, Pollard TD: Structure and function of the Arp2/3 complex. *Curr Opin Struct Biol* 1999, 9:244-249.
17. Kaverina I, Krylyshkina O, Small JV: Microtubule targeting of substrate contacts promotes their relaxation and dissociation. *J Cell Biol* 1999, 146:1033-1044.
18. Perez F, Diamantopoulos GS, Stalder R, Kreis TE: CLIP-170 highlights growing microtubule ends ***in vivo***. *Cell* 1999, 96:517-527.
19. Busson S, Dujardin D, Moreau A, Dompierre J, De Mey JR: Dynein and dynactin are localized to astral microtubules and at cortical sites in mitotic epithelial cells. *Curr Biol* 1998, 8:541-544.
20. Skop AR, White JG: The dynactin complex is required for cleavage plane specification in early ***Caenorhabditis elegans*** embryos. *Curr Biol* 1998, 8:1110-1116.

Supplementary material

Interaction of the p62 subunit of dynactin with Arp1 and the cortical actin cytoskeleton

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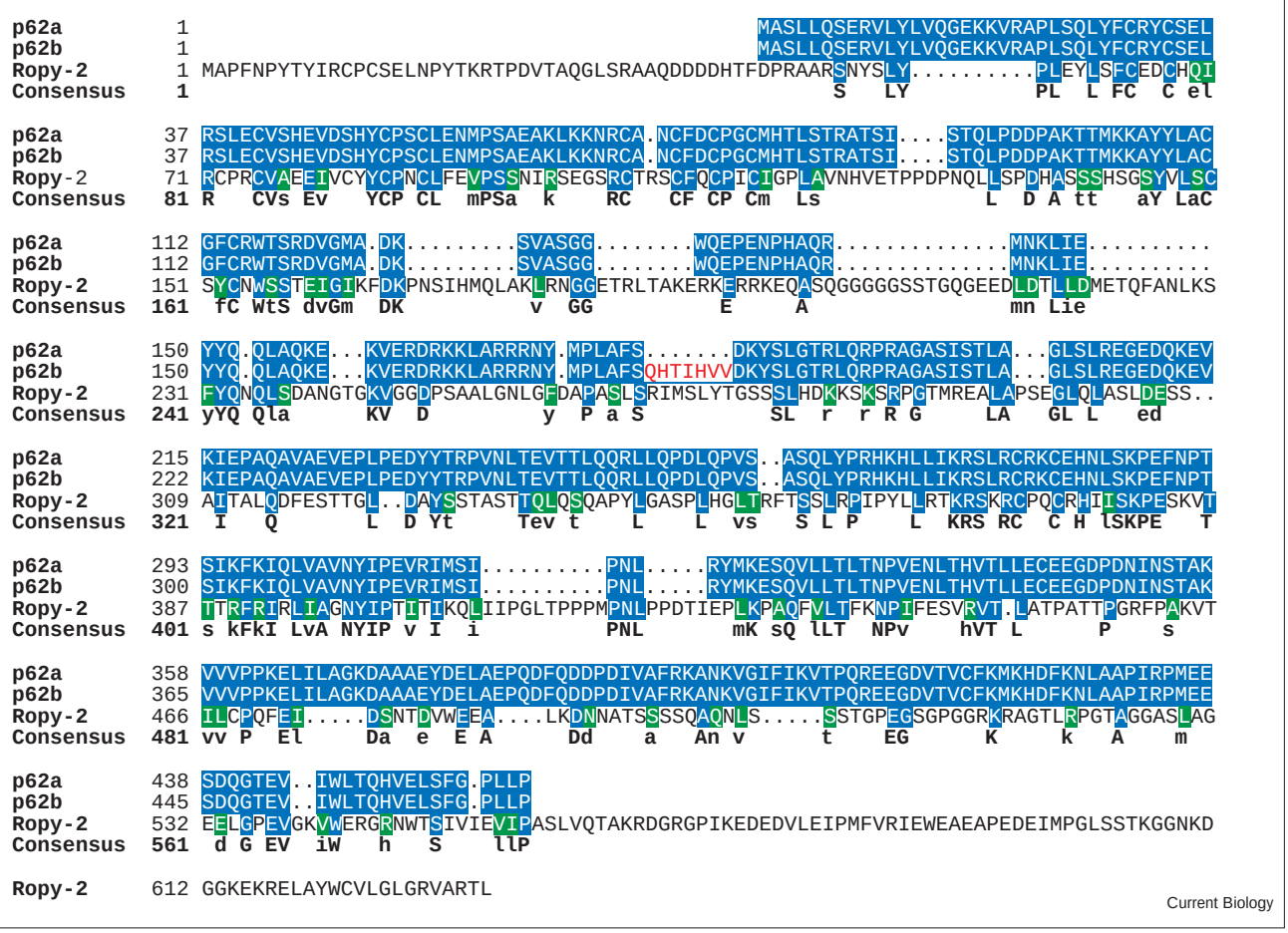
Figure S1



Alignment between our two rat brain p62 dynactin subunit isoforms and the *Neurospora* nuclear migration protein Ropy-2. Blue boxes highlight regions of amino acid identity and green boxes highlight areas of amino acid similarity. Red text shows amino acid insertion in isoform p62-b. The predicted open reading frame shown for Ropy-2 was obtained from Mike Plamann (personal communication).

Supplementary materials and methods

Dynactin purification

Dynactin was purified from a 150,000 × *g* supernatant of homogenized bovine brain tissue by 30% ammonium sulfate precipitation followed by centrifugation of the resuspended pellet through a 5–20% sucrose gradient. The 20S peak fractions were applied to a monoQ column, and dynactin was eluted using a linear 100–500 mM KCl gradient. Variable low amounts of dynein heavy chain were observed to copurify with dynactin using this procedure.

In vitro *translation and actin cosedimentation assays*

The p62–Myc protein was expressed using a T7-based coupled transcription/translation system (Promega) and radioactively labeled by the incorporation of [^{35}S]Met into the translation mix. The final 50 µl reaction mix was incubated at 30°C for 2 h, after which it was diluted into 1 ml of binding buffer (20 mM Tris pH 8.0, 150 mM KCl, 5 mM $MgCl_2$, 1 mM DTT) and then centrifuged at 100,000 × *g* for 1 h at 4°C. The resulting supernatant was incubated for 30 min at 37°C in the presence or absence of 1 µM skeletal muscle actin (Cytoskeleton Inc.) plus 1 µM phalloidin, and centrifuged at 350,000 × *g* for 15 min at room temperature. The supernatant and pellets (following three washes in binding buffer) were analysed by SDS–PAGE and subjected to autoradiography.

Immunological Reagents

Antibodies used in this study include monoclonal antibodies to the p150^{Glued} subunit of dynactin (Transduction Laboratories), β-galactosidase (Boehringer Mannheim), vinculin (Sigma), actin (AC15, Sigma), tubulin (DM1A, Amersham) and the Myc epitope (Boehringer Mannheim). Rabbit polyclonal antiserum (p62-Siskel) was generated against bacterially expressed p62-a. The A27 affinity-purified rabbit poly-

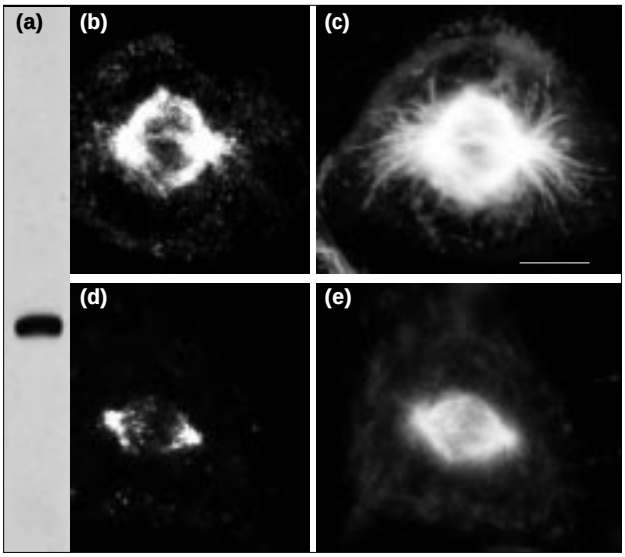
clonal antiserum [S1] was used to detect Arp1. Polyclonal anti-Myc antiserum was kindly provided by Melissa Gee. Texas-Red-conjugated secondary antibodies were generated in donkey and made species-specific by cross-adsorption (Jackson Immunoresearch Labs). Alexa Fluor 488 conjugated secondary antibodies were generated in goat and made species-specific by cross-adsorption (Molecular Probes).

Supplementary references

S1. Clark IB, Meyer DI: Overexpression of normal and mutant Arp1(alpha) (centractin) differentially affects microtubule organization during mitosis and interphase. *J Cell Sci* 1999, 112:3507-3518.

S2. Echeverri CJ, Paschal BM, Vaughan KT, Vallee RB: Molecular characterization of the 50-kD subunit of dynactin reveals function for the complex in chromosome alignment and spindle organization during mitosis. *J Cell Biol* 1996, 132:617-633.

Figure S2



Immunofluorescence microscopy of endogenous and overexpressed p62 in mitotic COS-7 cells. (a) A western blot of a COS-7 cell extract probed with p62-Siskel. (b,c) A pre-extracted and methanol fixed mitotic COS-7 cell overexpressing p62–Myc labeled with (b) a polyclonal anti-Myc antibody [S2] and (c) an anti-tubulin antibody (DM1A, Amersham). (d,e) Immunofluorescence localization of endogenous p62 at spindle poles in a mitotic COS-7 cell fixed in methanol and stained using (d) a rabbit polyclonal antibody (p62-Siskel) generated against bacterially expressed p62 followed by an Alexa Fluor 488 goat anti-rabbit IgG conjugate (Molecular Probes) and (e) anti-tubulin. Note that the p62 labeling also appears to spread along spindle microtubules.